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Distribution of Immunoreactive Thyrotropin-Releasing Hormone in the Brain and Hypophysis of Larval Bullfrogs with Special Reference to Nerve Fibers in the Pars Distalis

YUTAKA TANIGUCHI[1], SHIGEYASU TANAKA[2] and KAZUMASA KUROSUMI

Department of Morphology, Institute of Endocrinology, Gunma University, Maebashi 371, Japan

ABSTRACT—Thyrotropin-releasing hormone (TRH) was immunohistochemically detected in the brain and hypophysis of bullfrog larvae. At embryonic stage 24 (Shumway's classification), immunoreactive TRH was first detected in some fibers and perikarya in the hypothalamus, pars nervosa and pars intermedia. As metamorphosis proceeded, TRH-immunoreactive perikarya as well as fibers become conspicuous in several regions such as preoptic nucleus, infundibular nucleus, septum, amygdala and diagonal band of Broca. Appearance of immunoreactive TRH fibers in the pars distalis exclusively at Taylor-Korllos stage XIII-XVII was noted. The significance of this finding is discussed in relation to metamorphosis.

INTRODUCTION

Although thyrotropin-releasing hormone (TRH) has been purified and characterized early in the 1970's [1, 2], it was rather recent that the precise distribution of this peptide within the brain was elucidated. Leechan and Jackson [3] have succeeded in demonstrating TRH in the histological sections of the rat brain using acrolein as the fixative, and their protocol was applied to the tadpole brain by Mimnagh *et al.* [4]. We have investigated the development and localization of immunoreactive TRH neurons in the brain and hypophysis of larval bullfrogs, and found that a few immunoreactive TRH fibers appear in the pars distalis during a limited period of prometamorphosis. Siginificance of the temporal existence of the immunoreactive TRH fibers in the pars distalis will be discussed.

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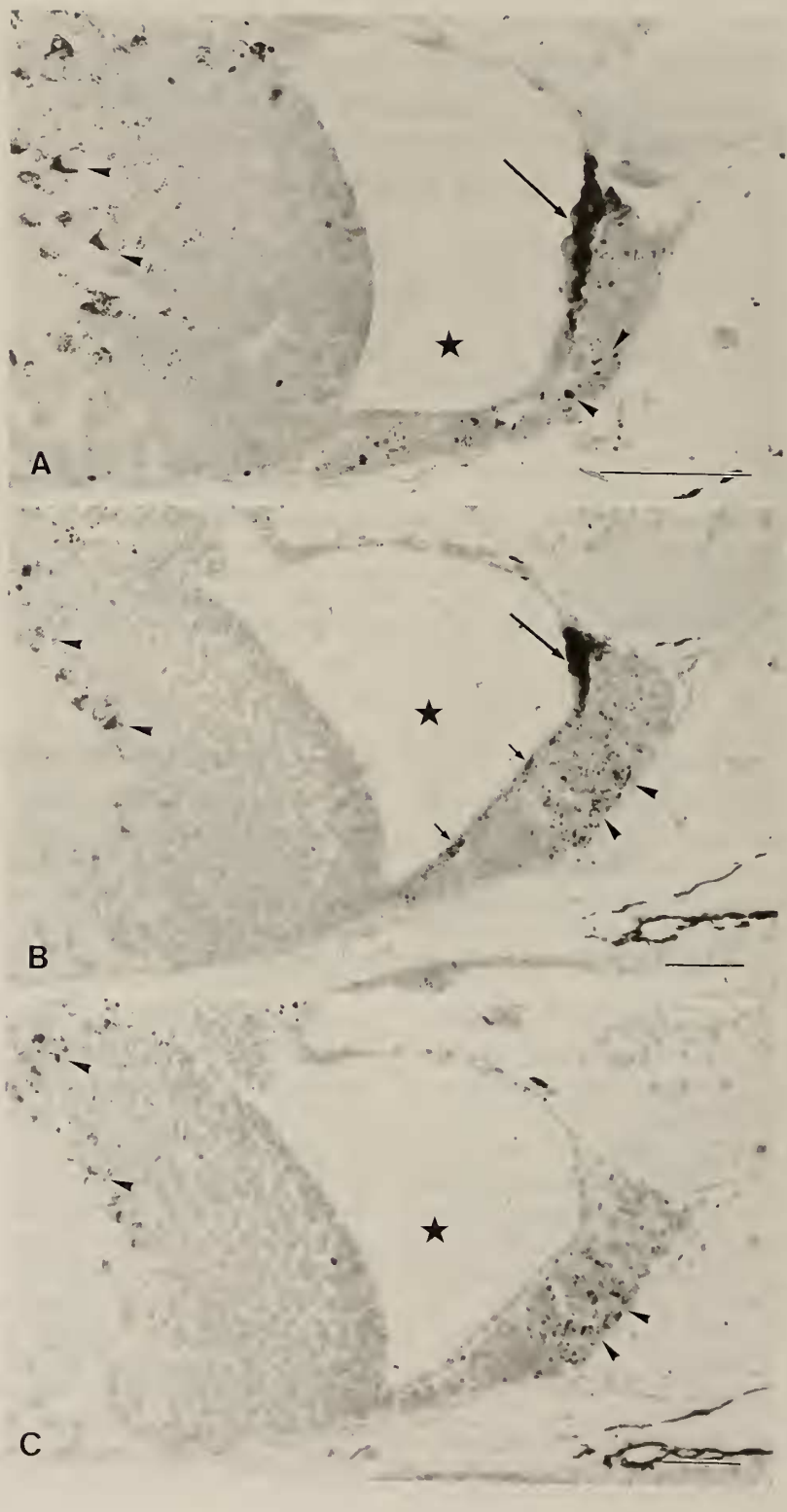
[1] Present address: Department of Anatomy, Wakayama Medical College, Wakayama 640, Japan.
[2] To whom all correspondence should be addressed.

MATERIALS AND METHODS

Larvae of the bullfrog (*Rana catesbeiana*) at various developmental stages were collected from the fields in the vicinity of Maebashi city. The animals were staged according to Shumway [5] for embryonic stages and Taylor and Kollros [6] for metamorphic stages. After acclimatization in a laboratory condition for 20 days, the animals were decapitated and the brain with hypophsis was carefully removed from the skull and fixed in 5% acrolein in 1/15 M phosphate buffer (pH 7.4) for 3 hr at room temperature. The tissue was embedded in paraplast after the dehydration with ethanol series. Serial sagittal 4 μm-thick sections were cut and immunohistochemically stained according to the method of Leechan and Jackson [3] with minor modifications. The deparaffinized sections were treated first with 10 mM sodium metaperiodate in phosphate-buffered saline (PBS) for 1.5 min, followed by PBS wash. Then they were treated with 1% sodium borohydride in PBS for 1.5 min, followed by thorough wash with PBS. Then the sections were stained by the peroxidase-anti-peroxidase (PAP) method: normal goat serum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(1 : 20) for 2 hr, rabbit anti-TRH serum (1 : 3000) for 14–18 hr, PBS wash, goat anti-rabbit γ-globulin (1 : 200) for 2 hr, PBS wash, PAP complex (1 : 200) for 2 hr, PBS wash. The reaction product was visualized with 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 for 5–10 min. After washing with distilled water, the sections were stained with Methyl green, dehydrated with an ethanol series, and mounted with Eukitt.

To check the specificity of the immunostaining, the diluted anti-TRH serum was preabsorbed with TRH at a final concentration of 10 μg/ml overnight at 4° C prior to its use in the immunohistochemistry. The anti-TRH serum was a kind gift from Dr. M. Mori, School of Medicine, Gunma University [7], and PAP complex was purchased from Dakkopats, Denmark.

RESULTS

No immunoreactive TRH was detected in the brain of the specimens at earlier stages than embryonic stage 24 (body length ca. 8 mm). TRH immunoreactivity was first demonstrable in the brain and pars nervosa at stage 24 (Fig. 1A). In the brain, immunoreactive TRH nerve fibers were found in the medial preoptic area and in the region of infundibular nucleus. Some neuronal perikaya in the region of putative preoptic nucleus were also TRH-positive at this stage. In the hypophysis only the putative pars nervosa was immunostained with anti-TRH serum. At stage 25 (body length ca. 13 mm), immunoreactive TRH fibers ascending from the ventral hypothalamus to the pars nervosa were observed (Fig. 1B). The pars nervosa was diffusely immunostained with anti-TRH serum from this stage onwards. As metamorphosis proceeds, the immunoreactive TRH structures became more widely distributed in the brain, including preoptic nucleus, infundibular nucleus, septum, amygdala and diagonal band of Broca, and the intensity of the staining was increased (Fig. 2). Similarly, in

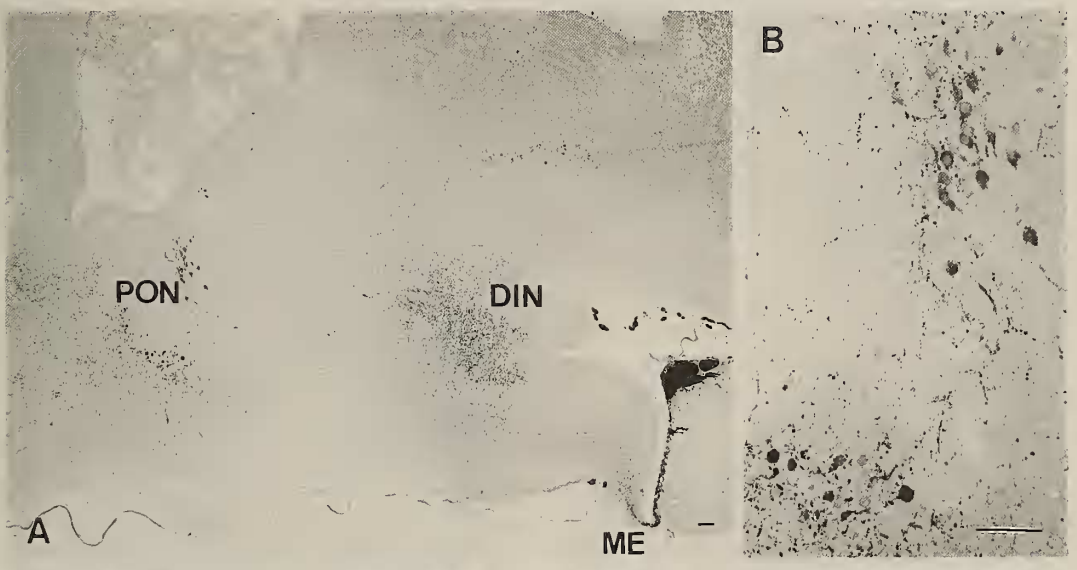

FIG. 2. Sagittal section of brain in bullforg tadpoles at stage XIII immunostained with anti-TRH serum. Many perikarya and fibers were observed in the dorsal portion of preoptic nucleus (PON) (Fig. 2B). In the dorsal infundibular nucleus (DIN), a dense fiber network were found. ME: Median eminence. Bar: 50 μm.

FIG. 1. Sagittal sections of embryonic brain and hypophysis in bullfrogs immunostained with anti-TRH serum. The star indicates the third ventricle. The putative pars nervosa (arrow) was first stained at stage 24 (A). At stage 25 (B), in addition to the pars nervosa (arrow), some fibers are observed to ascent the future median eminence and infundibular floor (short arrows). When adjacent section of B was immunostained with the preabsorbted anti-TRH serum, immunoreactivity was completely abolished (C). Note that embryonic cells contain much melanin pigment (arrowhead). Bar: 50 μm.

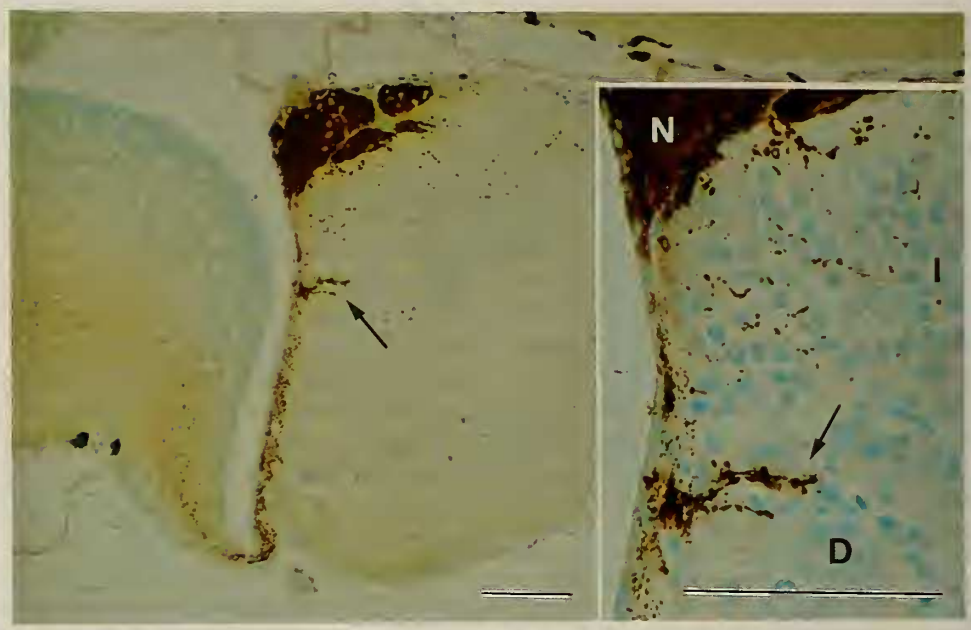

FIG. 3. Sagittal sections of hypophysis in bullfrog tadploes at stage XIII immunostained with anti-TRH serum. The fibers are sometimes found to penetrate into the pars distalis (arrow) as shown in the inset. D: Pars distalis; I: Pars intermedia; N: Pars nervosa. Bar: 50 μm.

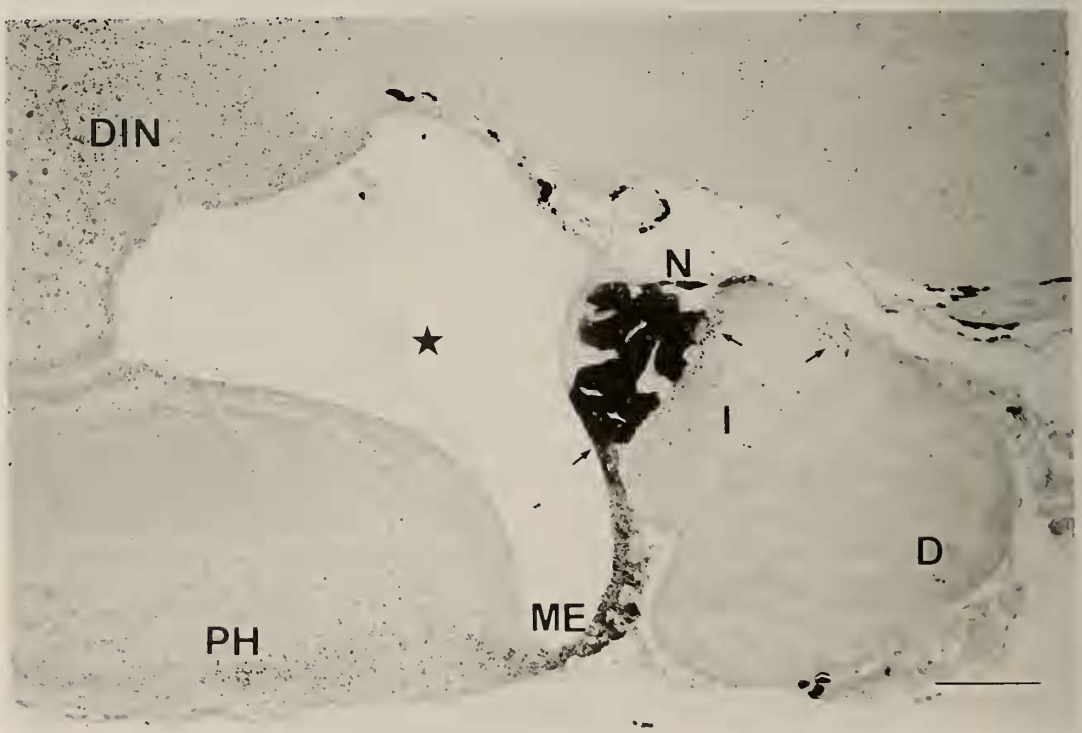

FIG. 4. A sagittal section of bulfrog hypophysis after metamorphosis completed (stage XXV). No TRH-fibers are observed in the pars distalis (D), though in the pars intermedia (I) and in the pars nervosa (N) the immunostaining remains as before (arrow). DIN: Dorsal infundibular nucleus; ME: Median eminence; PH: Preoptic-hypophysial tract. Bar: 100 μm.

the pars nervosa the immunoreactivity became intense. In the pars intermedia, numerous immunoreactive TRH fibers were observed from stage 24 througout the process of metamorphosis. Although embryonic cells contained much melanin pigment, the reaction product of immunostaining was easily distinguishable from the melanin granules by the difference in their colors. Moreover, as shown in Figure 1C, these immunoreactions were completely abolished after preabsorption of the anti-TRH serum. Careful observations revealed that at stages XIII-XVII in prometamorphosis, a few immunoreavtive TRH fibers penetrated into the pars distalis from the median eminence as well as from the pars intermedia (Fig. 3). These immunoreactive TRH fibers in the pars distalis disappeared at the onset of metamorphic climax (stage XX). In the larvae beyond stage XX, TRH-immunoreactivity in the hypophysis was confined to the pars nervosa and pars intermedia (Fig. 4).

DISCUSSION

The present study demonstrated the localization of immunoreactive TRH in the brain and hypophysis of larval bullfrog during metamorphosis. During metamorphosis, immunoreactive TRH was found in the pars nervosa and pars intermedia, and in some brain areas including preoptic nucleus, infundibular nucleus, septum, amygdala and diagonal band of Broca. This histological distribution of immunoreactive TRH was essentially similar to that reported previousely [4, 8, 9]. Moreover, the present study demonstrated clearly that immunoreactive TRH fibers penetrate into the pars distalis at prometamorphic stage. To our knowledge, the presence of TRH fibers in the pars distalis has not been described in the bullfrog or other vertebrates throughout larva and adult. The discrepancy between the present study and others may be ascribed to the technical differences. Previous investigators used the mixture of formaldehyde and glutaraldehyde as the fixative and thick frozen sections, while we used acrolein fixative and thin-paraplast sections. Leechan and Jackson [3] have reported that TRH in the tissue is well preserved only with acrolein. Moreover, the employment of thick frozen sections may get into difficulty to reveal the precise distribution of immunoreactive TRH fibers in the hypophysis.

Aronsson [10] has demonstrated monoaminergic nerve fibers in the pars distalis of *Rana temporaria* by Falck-Hillarp method and stated that the presence of these fibers was confined to the stages from prometamorphosis to just before the climax. Kawakami-Kondo *et al.* [11] have reported the tyrosine hydroxylase-positive nerve fibers in the pars distalis which was confined to the Gosner's stage 31 to 40. The time of appearance and disappearance of these monoaminergic or dopaminergic fibers in the pars distalis was nearly identical to those of the immunoreactive TRH fibers in the present study.

In the teleost hypophysis, some nerve endings exist in the pars distalis [12]. In higher vertebrates, indirect connection by way of the hypophysial portal vessels was predominantly seen, although nerve fibers were occasionally observed in the pars distalis in rats [13]. The immunoreactive TRH- and monoamine-containing fibers in the pars distalis during metamorphosis might be considered as an intermediate type between the teleost and the higher vertebrate.

In amphibian larvae at premetamorphic and early prometamorphic stages, the pars distalis is in close contact with the median eminence where the capillary loop is poorly developed. Penetration of the capillary into the median eminence takes place as metamorphosis progresses. At climax, development of the median eminence is completed and the pars distalis almost detaches from the median eminence, being connected with it by the portal vessel at the rostral region [14]. This morphological change in the hypothalamus-hypophysial complex, which also observed in the present experiment, may have something to do with the disappearance of the immunoreactive TRH fibers from the pars distalis. Considering that the hypophysial portal vessels are still in the process of formation during the period of the existence of the immunoreactive TRH fibers in the pars distalis, the immunoreactive TRH may temporally function as a controling factor of secretion of a certain hypophysial hormone(s) by "paracrine mechanism" which is involved in metamorphosis [see, 15,

16]. However, no definite conclusion can be drawn since physiological role of TRH in controling hypophysial function in amphibian larvae is not clear.

The effect of mammalian TRH on hypophysis-thyroid axis in the amphibians has been controversial. It has been generally thought that TRH has no thyrotropin-releasing effect both *in vivo* and *in vitro* [17–21]. However, recent reports indicate that TRH increases thyroxine levels in the frog [22, 23]. Therefore, a direct evidence that TRH induces thyrotropin release is awaited. On the other hand, it is evident that TRH stimulates the release of prolactin from the pars distalis *in vitro* [24–26] and *in vivo* [27]. Further studies on the identification of cell type(s) of the pars distalis around which the immunoreactive TRH fibers terminate may give on insight into the physiological role of TRH in the regulation of hypophysial hormone secretion during metamorphosis.

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